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Multiparametric Analysis of Apoptosis by Flow and Image Cytometry

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Summary

Flow cytometric assays for apoptosis are now in widespread use. The multiparametric nature of flow cytometry allows multiple assays for several apoptotic characteristics to be combined in a single sample, providing a powerful tool for elucidating the complex progression of apoptotic death in a variety of cell types. This chapter describes one such assay, allowing simultaneous analysis of caspase activation, annexin V binding to "flipped" phosphatidylserine residues and membrane permeability to DNA binding dyes. This multidimensional approach to analyzing apoptosis provides far more information than single-parameter assays that provide only an ambiguous "percent apoptotic" result, given that multiple early, intermediate, and late apoptotic stages can be visualized simultaneously. This multiparametric approach is also amenable to a variety of flow cytometric instrumentation, both old and new.

Key Words

7-Aminoactinomycin D, annexin V, apoptosis, caspase, flow cytometry, propidium iodide.

1. Introduction

The importance of apoptosis in the regulation of cellular homeostasis has mandated the development of accurate assays capable of measuring this process. Apoptosis assays based on flow cytometry have proven particularly useful; they are rapid, quantitative, and provide a individual cell-based mode of analysis (rather than a bulk population) ***(1)***. The multiparametric nature of flow cytometry also allows the detection of more than one cell death characteristic to be combined in a single assay. For example, apoptosis assays that utilize DNA dyes as plasma membrane permeability indicators (such as propidium iodide [PI]) can be combined with assays that assess different cellular responses associated with cell death, including mitochondrial membrane potential and

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annexin V binding to "flipped" phosphatidylserine (PS) ***(2–4)***. Combining measurements for cell death into a single assay has a number of important advantages; it provides simultaneous multiple confirmation of apoptotic activity (important in a process that has proven highly pleiotrophic in phenotype). It also provides a much more comprehensive and multidimensional picture of the entire cell death program.

Recognition of the pivotal role of caspases in the death process has led to the recent development of assays that can measure these important enzymes *in situ*. Caspase activation represents one of the earliest measurable markers of apoptosis ***(5)***. In most cases, caspase activation precedes degradation in cell permeability, DNA fragmentation, cytoskeletal collapse, and PS "flipping," and is likely important in triggering these later manifestations of cell death. Combining fluorogenic assays for caspase activity with fluorescence-based assays for later manifestations of cell death (such as membrane alterations and loss of membrane permeability) can provide a very information-intensive view of cell death, particularly in its early stages (when most of the relevant biochemical signaling activities occur and are likely to still be intact prior to complete cell structural collapse) ***(6–10)***.

Several fluorogenic assays for caspase activity have been described in the literature, including the OncoImmunin PhiPhiLux system and the FLICA substrates ***(11–16)***. In this chapter, we describe the integration of the PhiPhiLux caspase substrate system with two simultaneous assays for later manifestations of cell death, namely annexin V binding to "flipped" PS residues and cell membrane permeability to a DNA binding dye ***(16)***. The PhiPhiLux caspase substrates have several notable characteristics; they are cell permeable, demonstrate relatively good caspase specificity, possess high signal-to-noise ratios between their uncleaved and cleaved forms, and have fluorescence spectral properties that are compatible with other fluorescent probes ***(14–16)***. The ability to measure three apoptotic phenotypes in a single assay provides a powerful and comprehensive view of the apoptotic process, applicable to both suspension cells by traditional flow cytometry, and adherent cells using laser scanning cytometry ***(16)***. This assay can be adapted to both current and older flow cytometers.

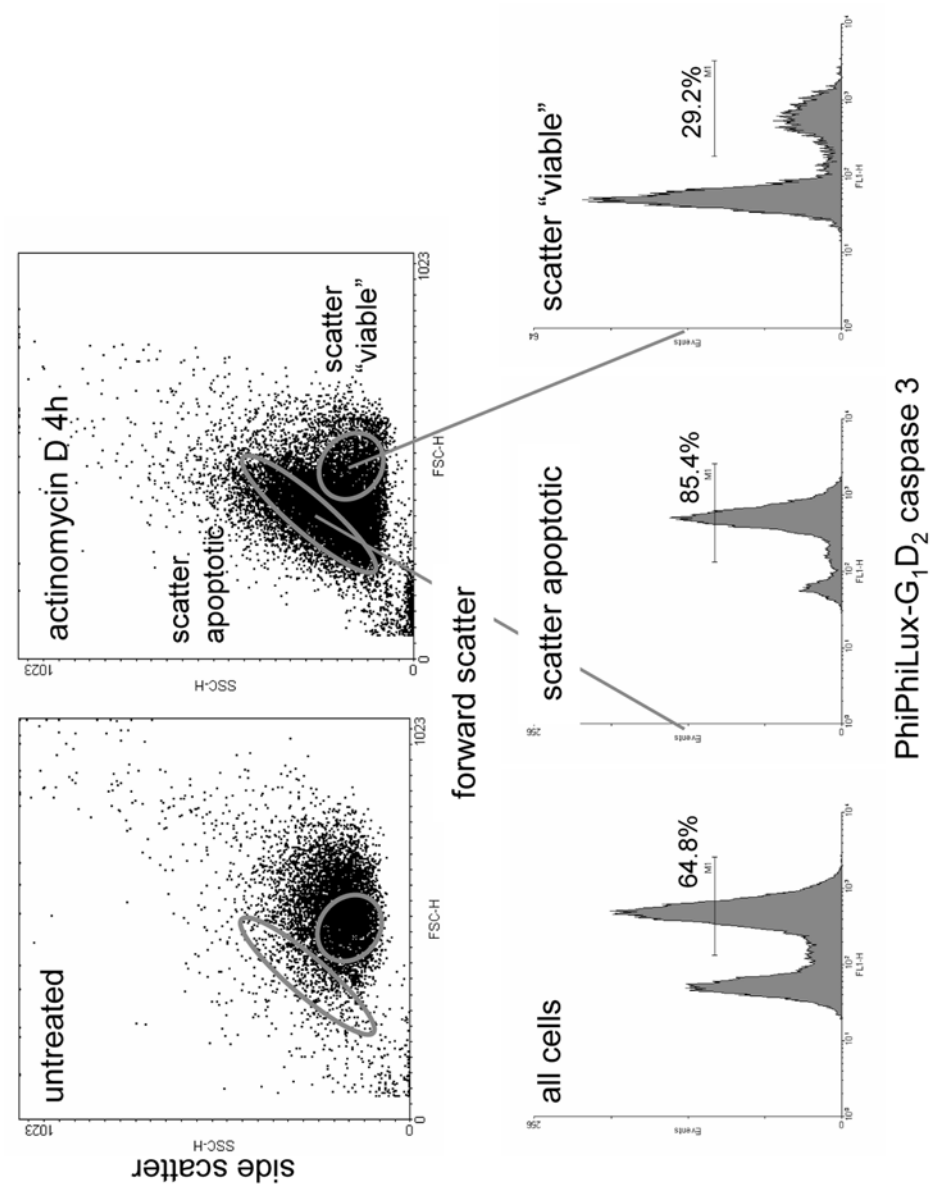
2. Materials

1. PhiPhiLux-G_1D_2 fluorogenic caspase 3 substrate (OncoImmunin, Inc., Gaithersburg, MD): OncoImmunin makes a wide variety of fluorogenic enzyme substrates that become fluorescent upon cleavage. The fluorogenic caspase 3/7 substrate (termed PhiPhiLux-G_1D_2) consists of an 18-amino-acid peptide constituting the recognition and cleavage sequence from poly(ADP-ribose) polymerase (PARP), a physiological target for caspase 3 ***(17)***. The substrate is homodoubly labeled with fluorophores (in this case, one similar in properties to fluorescein) on opposite

sides of the molecule; in this conformation, the fluorochrome molecules are in close physical proximity (<15 Å), and the fluorescence of the resulting complex is largely quenched ***(15,18)***. After the substrate enters a cell by passive diffusion and is cleaved by caspase 3 (or 7), the unquenched fluorescent fragments will be retained on the side of the membrane where the cleavage took place. This is attributable to the fact that these singly labeled peptides have very low membrane permeability as compared with the intact substrate, allowing the cleaved fragments to remain in the cell for several hours ***(15,18)***.

a. PhiPhiLux-G_1D_2 can be excited with a standard 488-nm laser found on most flow cytometers, and is spectrally compatible with PI or 7-aminoactinomycin D (7-AAD) (which will be subsequently used for measuring apoptotic cell permeability) and either phycoerythrin (PE)- or allophycocyanin (APC)-conjugated annexin V (for detection of PS "flipping" during apoptotic death).
b. The PhiPhiLux reagents are largely nonfluorescent in the uncleaved state and is extremely bright on caspase activation. The expected signal-to-noise ratio between unlabeled and substrate-loaded viable and apoptotic cells is shown in **Fig. 1**, where actinomycin D-treated EL-4 thymoma cells were labeled with PhiPhiLux G_1D_2 and analyzed by flow cytometry; the apoptotic cells possess one to three orders of magnitude higher fluorescence than the presumably viable cell population. It should be noted that primary cell cultures may show somewhat lower levels of caspase activation than cell lines, with subsequent lower levels of substrate fluorescence (*see* **Note 1**).
c. The PhiPhiLux reagents are also available with other fluorescent tags, including a rhodamine-like fluorochrome (requiring a green excitation source, not commonly available on commercial flow cytometer systems), and a proprietary Cy5-like fluorochrome that can be excited with a red laser. While the rhodamine-conjugated caspase substrates are not commonly used for flow cytometry because of their excitation requirements, the Cy5-like substrate can be used on flow cytometers equipped with a red laser source. They can also be combined with the fluorescein-like PhiPhiLux-G_1D_2 substrate for detection of multiple caspases in a single assay, as shown in Fig. 4.
d. The PhiPhiLux reagents are commercially provided at concentrations of 5–10 μ*M* in sealed aliquots and can be stored at 4oC prior to opening; once the ampule is opened, any remaining substrate should be stored at –20°C. Avoid repeated freezing and thawing.

2. PE- or APC-conjugated annexin V (e.g., Caltag Laboratories, Burlingame, CA): Annexin V is available conjugated to a variety of fluorochromes, and binds tightly to apoptotic cells with "flipped" PS residues on their extracellular membrane leaflet. It should be noted that damaged or necrotic cells with a high degree of membrane permeability will also bind annexin V to their intracellular membrane leaflet, despite their questionable apoptotic nature; therefore, a DNA binding dye as a cell permeability indicator should always be incorporated into annexin V binding assays.
3. PI: PI is an intercalating DNA binding dye available from a wide variety of sources. It excites at 488 nm and emits in the 570–630 nm range. It should be


side scatter
SSC-H
FSC-H
untreated
actinomycin D 4h
scatter apoptotic
scatter "viable"
forward scatter
all cells
64.8%
scatter apoptotic
85.4%
scatter "viable"
29.2%
PhiPhiLux-G1D2 caspase 3

dissolved in deionized water at 1 mg/mL and stored in the dark at 4°C for up to 3 mo.

4. 7-aminoactinomycin D (Sigma Chemical Co., St. Louis, MO or Molecular Probes, Eugene, OR): 7-AAD is a DNA binding dye that excites at 488 nm and emits in the far red, with an emission peak at approx 670 nm. 7-AAD can be used as an alternative to PI where a longer wavelength cell permeability probe is desired. 7-AAD should be dissolved in 95% ethanol at 1 mg/mL and stored at –20°C. Solubilized stocks are good for three months. Diluted stocks should be used within 24 h. *See* **Note 2**.
5. Wash buffer: Dulbecco's phosphate-buffered saline (PBS) (containing calcium and magnesium) supplemented with 2% fetal bovine serum. This is used for cell washing prior to DNA dye addition.
6. Complete medium: RPMI-1640 and 10% fetal bovine serum.

3. Methods

3.1. Combinations of Fluorochromes

This assay introduces fluorescent labels for three characteristics of cell apoptosis: caspase activation, PS "flipping," and cell permeability. There is some flexibility of fluorochrome selection for the investigator depending on the flow cytometric instrumentation available. Two possible combinations are described below, one for analysis on instruments equipped with a single 488-nm argon-ion laser, and the second on instruments equipped with dual 488 nm/red diode or red helium–neon (He–Ne) lasers.

1. Single 488-nm laser instruments: Examples of these include the Becton-Dickinson FACScan (or single laser FACSort or FACSCalibur) (BD Biosciences, San Jose, CA), and the Beckman Coulter XL (Beckman Coulter, Miami, FL) flow cytometers. The following combination should be used when analysis is limited to this instrument type:
   a. PhiPhiLux-G_1D_2 (similar to fluorescein): This fluorochrome will occupy the fluorescein isothiocyanate (FITC) detector channel on most commercial instruments.
   b. PE-conjugated annexin V: This fluorochome will be detected in the phycoerythrin channel of most instruments. Some fluorescent compensation will be required to separate the PE signal from PhiPhiLux-G_1D_2 and 7-AAD.

Fig. 1. *(see facing page)* Caspase activation in apoptotic cells. EL-4 cells were incubated with no treatment or with actinomycin D at 5 μg/mL for 4 h, followed by labeling with PhiPhiLux-G_1D_2. **Top** dot plots show forward vs side scatter profiles for both untreated and drug-treated cells. **Lower** histograms show caspase activation distributions for all actinomycin D-treated cells **(left)**, scatter apoptotic cells only **(middle)**, and scatter "viable" cells **(right)**.

c. 7-AAD: This far-red emitting DNA binding dye can be detected in the far red (or PE-Cy5) detector of most commercial instruments.

2. Dual 488-nm/red laser-equipped instruments: Several more recent benchtop flow cytometers are equipped with more than one laser, most commonly a red source (such as a 635-nm red diode or 633-nm red He–Ne laser). The Becton-Dickinson FACSort, FACSCalibur, LSR and LSR II fall into this category, as does the Beckman Coulter FC500. A red laser allows several red-excited fluorochromes to be incorporated into flow cytometry assays, including APC. Another group of PhiPhiLux caspase substrates incorporating a proprietary red-excited fluorochrome analogous to Cy5 can also be used on these instruments. The following combination is suggested for dual-laser instrumentation:
   a. PhiPhiLux-G_1D_2 (similar to fluorescein): This fluorochrome will occupy the FITC detector channel on most commercial instruments.
   b. APC-conjugated annexin V: This fluorochome can be excited with either red diode or He–Ne lasers and emits in the far red range. It requires little fluorescent color compensation to separate its signal from PhiPhiLux-G_1D_2 or the DNA binding dyes described in **step2**.
   c. PI or 7-AAD: Both of these DNA binding dyes can be incorporated into a cell death assay with PhiPhiLux-G_1D_2 and APC-annexin V. Both are detected through the far red detector channel (usually with a mid-600-nm bandpass or longpass filter) on most flow cytometers.
3. Other fluorochrome combinations: Multilaser flow cytometers with unusual wavelength laser sources are becoming more common in flow cytometry. Similarly, the number of fluorochromes available for flow cytometry is increasing exponentially. Other fluorochome combinations for the above assays are certainly possible and can be considered by the experienced investigator, particularly if additional fluorescence-based apoptotic assays are to be incorporated into the assay described in **Subheading 3.6.**

3.2. Preparation of Cells

1. Harvest suspension cell lines or cultured primary cells; transfer to 12 × 75 mm cell culture tubes.
2. Centrifuge cells at 400*g* for 5 min and decant supernatant. Nearly complete removal of the supernatant is critical for the steps that follow; the amount of remaining supernatant should be as low as possible. Although cells can be washed prior to labeling, performing the assay in the remaining complete media supernatant will reduce the amount of cell death occurring during the assay. If cells are obtained from clinical or other in vivo sources, they should be centrifuged and resuspended in complete medium prior to use, then centrifuged and decanted as described above.
3. Label 0.5–2 × 10^6 cells per sample; increasing this number will reduce caspase and annexin V labeling efficiency. Adherent cells pose special challenges for apoptotic analysis due to the trauma association with cell dissociation; analysis in the

adherent state by laser scanning cytometry is preferable to suspension flow cytometry under these circumstances (*see* **Note 3**).

4. EL-4 cells treated with actinomycin D at 5 µg/mL for 4 h were used to illustrate this assay in **Figs. 1–4**. This cell system also can make a useful positive control for more general use. *See* **Note 1**.

3.3. Fluorogenic Caspase Substrate Labeling

Cells are initially loaded with the PhiPhiLux caspase substrate. Substrate concentration and incubation time are critical factors in cell-permeable substrate labeling.

1. Tap each tube to resuspend the cell pellet in the remaining supernatant. The supernatant in the tubes will be approx 50 µL in volume.
2. Add 50 µL of the PhiPhiLux reagent to each tube and shake gently. The PhiPhiLux reagent should be diluted as little as possible for maximum detection, hence the need for minimal sample supernatant. For an initial PhiPhiLux reagent solution concentration of 10 µ*M*, this will give a final concentration between 3 and 5 µ*M*. For optimal labeling, the PhiPhiLux reagent can be titered between 0.5 and 5 µ*M*, although this should be done with caution (*see* **Note 4**).
3. Incubate the tubes for 45 min at 37°C.

3.4. Annexin V Labeling

Cells are now labeled with fluorochrome-conjugated annexin V. Since centrifuge washings are minimized in this method to reduce assay-associated cell death, the cells are not washed following caspase substrate loading but are labeled immediately with fluurochrome-conjugated annexin V. Because most cell culture media and serum supplements contain calcium and magnesium, it is assumed that cation concentrations are sufficient to allow annexin V binding to "flipped" PS residues. Subsequent cell washing is done in wash buffer (containing calcium and magnesium) to maintain annexin V binding. *See* **Note 5**.

1. Remove the above tubes after 45 min of incubation and add the appropriate fluorochrome-conjugated annexin V (in this case, either PE or APC). Annexin V is generally available in suspension at concentrations ranging from 0.1 to 1 mg/mL. Cell labeling should be carried out at approx 0.5–5 µg annexin V per sample. Therefore, 0.5–5 µL of a 1 mg/mL annexin V solution solution would be added to the above tubes (now at approx 100–150 µL total volume). Again, fluorochrome-conjugated annexin V labeling should be titered in advance of actual use.
2. Incubate at room temperature for 15 min.
3. Add 3 mL of the wash buffer to each tube. Centrifuge at 400*g* for 5 min, and decant the supernatant.

3.5. DNA Binding Dye Labeling

Depending on the instrumentation available, cells can be subsequently labeled with either PI or 7-AAD for assessment of cell permeability in the later stages of apoptosis ***(6)***. PI should be used with dual laser instruments, as it is spectrally compatible with both the PhiPhiLux-G_1D_2 substrate and APC; while it can be used with PE, the significant spectral overlap between the two fluorescent molecules makes this inadvisable. The DNA binding dye 7-AAD can be used with either single- or dual-laser instrument configurations, as it is spectrally compatible with all of the above fluorochromes. (*See* **Note 2**.)

1. Prepare a solution of either PI at 2 µg/mL or 7-AAD at 5 µg/mL in complete medium.
2. Once the above tubes are decanted, add 0.5 mL of either the PI or 7-AAD solution. Samples should then be maintained at room temperature and *analyzed within 60 min. See* **Note 6**.

3.6. Flow Cytometric Analysis

Cells should be analyzed as quickly as possible to minimize post-assay apoptotic death. The instrument should be set up and ready for sample acquisition immediately on completion of the assay. The choice of fluorescent reagents for both single- and dual-laser flow cytometers is described above; fluorescent detector assignments and analysis issues are described here.

1. PhiPhiLux-G_1D_2: This fluorescein-like caspase substrate is analyzed through the fluorescein or FITC channel on most flow cytometers (often with the arbitrary designation of "FL1"), usually equipped with a 530/30 nm or similar narrow bandpass filter. The spectral properties of the PhiPhiLux-G_1D_2 fluorochrome is similar to fluorescein, requiring some color compensation when used simultaneously with PE or PI (and to a lesser extent with 7-AAD).
2. PE-conjugated annexin V: Like most PE-conjugated reagents, this reagent is detected through the PE channel on most cytometers (frequently with the designation "FL2"), usually equipped with a 575/26-nm or similar bandpass filter. PE requires color compensation when used with PhiPhiLux-G_1D_2 and 7-AAD.
3. APC-conjugated annexin V: APC is excited with a red laser source and detected through the APC channel on many flow cytometers (sometimes with an "FL4" designation) using a 660/20-nm or similar bandpass filter. An advantage of APC in multicolor assays is its minimal need for color compensation; there is no significant spectral overlap between PhiPhiLux-G_1D_2, PI, or 7-AAD.
4. PI: This DNA binding dye is extremely bright even at low concentrations, and has a broad emission range, necessitating compensation when used with PhiPhiLux-G_1D_2. It can be detected in either the PE or far red detection channel (with either the PE 575/26-nm filter or a longer red optic), with the latter choice being preferable to reduce spillover into the fluorescein detector.

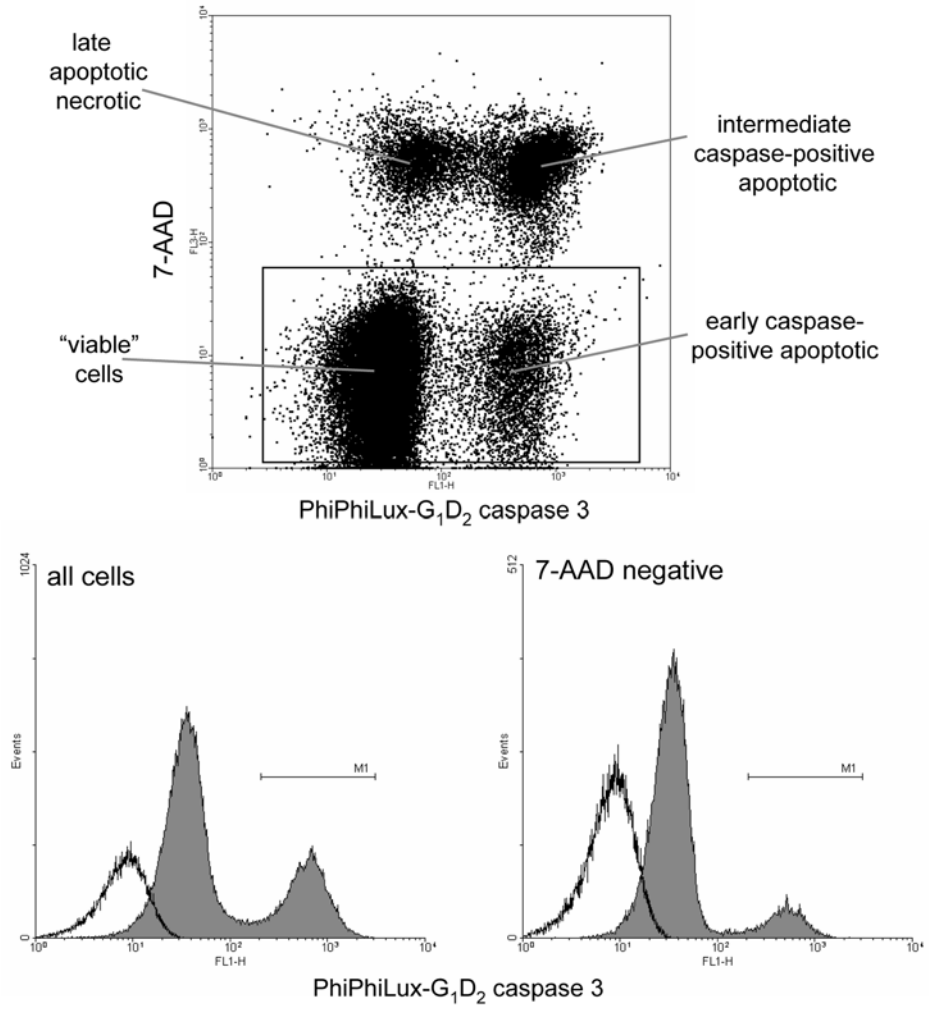


Fig. 2. Caspase activation and 7-AAD permeability in apoptotic cells. EL-4 cells were incubated with actinomycin D at 5 µg/mL for 4 h, followed by labeling with PhiPhiLux-G_1D_2 and 7-AAD at 5 µg/mL. **Top** dot-plot shows caspase activation vs 7-AAD permeability protocols, with the four major populations of "viable" and apoptotic cells identified. **Lower** histograms show caspase activation distributions for all cells **(left)** and 7-AAD-negative cells, derived from the indicated cytogram gate **(right)**. Cell controls with no PhiPhiLux-G_1D_2 loading are shown in the open histogram peaks.

5. 7-AAD: This DNA binding dye is dimmer than PI and emits in the far red, allowing its detection in the far red channel on most flow cytometers (often designated "FL3") with a 675/20-nm bandpass or 650-nm longpass dichroic or similar filter. Compensation will be required when used with PhiPhiLux G_1D_2 and PE. 7-AAD

has an unexplained ability to slightly quench PE fluorescence in some multicolor assay systems, so this should be taken into account during instrument setup.

3.7. Gating for Flow Cytometry

Gating at several levels is critical for meaningful analysis of apoptosis. A typical gating scheme is illustrated in **Fig. 3** and is described in more detail in **Subheading 3.9.** Some general guidelines are listed here.

1. Scatter gating: Many cell lines and some primary cells show a dramatic alteration in forward and side scatter measurements (rough indicators of cell size and optical density, respectively) late in the onset of apoptosis. It is therefore tempting to draw a gate around the scatter "viable" population, and look at caspase activation, annexin V binding and DNA dye uptake in these cells alone as measures of early cell death. However, there is considerable evidence that the scatter apoptotic population (shown in **Fig. 1**) may in fact contain some viable cells that have undergone a transient shift in cell volume well prior to other "early" markers of cell death such as caspase activation. It is therefore advisable to gate the entire scatter population (excluding obvious debris) for the most accurate analysis of cell death. Subsequent gating of the "viable" population (which may in fact contain early apoptotic cells) can be done at the level of annexin V binding and DNA dye incorporation.
2. Annexin V binding and DNA binding dye exclusion: These markers usually occur after caspase activation and are considered "later" markers of apoptosis. Therefore, annexin V and DNA binding dye positive and negative subpopulations can be gated for discrimination of "early" and "late" apoptotic cells. Subsequent analysis of caspase activation in these subsets can further delineate the early stages of cell death, as illustrated in **Fig. 3**.
3. PI vs 7-AAD: As pointed out in **Note 2**, PI and 7-AAD are not completely interchangeable with regard to exclusion by apoptotic cells; 7-AAD is somewhat more cell permeable than PI and will label an earlier subset of apoptotic cells. If 7-AAD-positive cells are excluded from the analysis (in an attempt to quantify very early apoptotic events), this greater cell permeability will result in a lower

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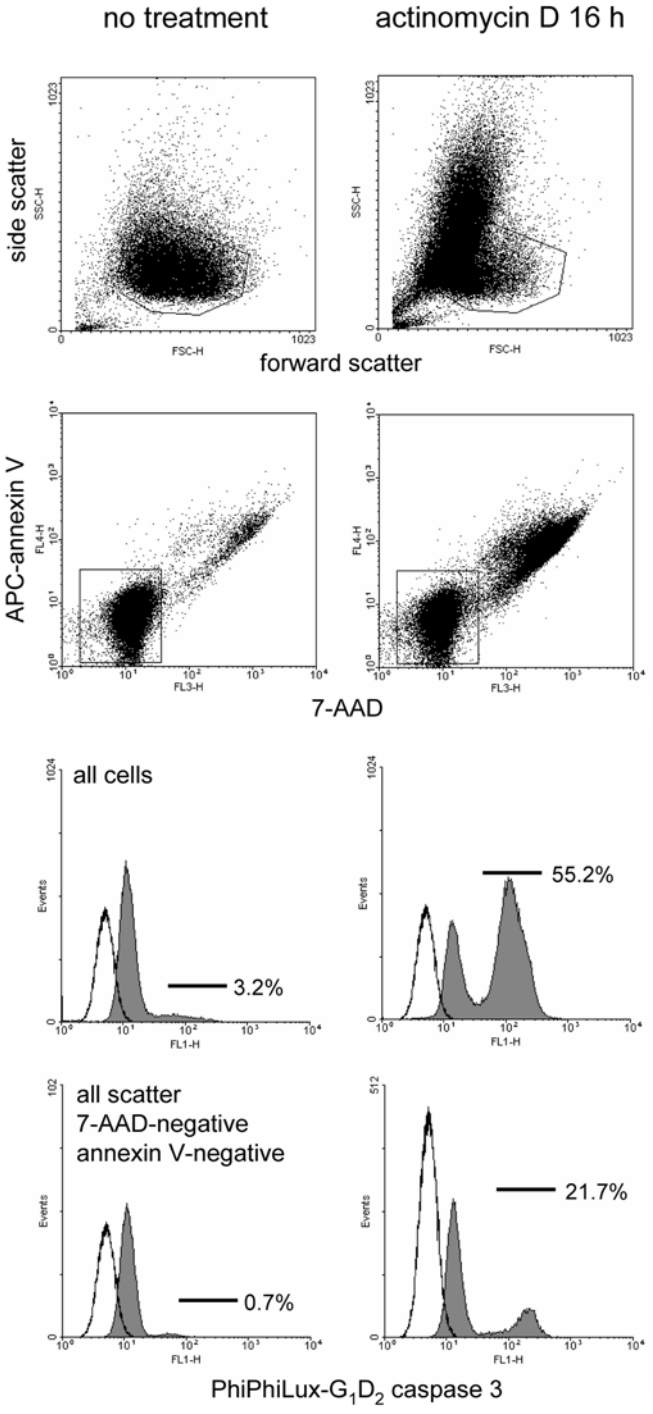
Fig. 3. *(see facing page)* Caspase activation, annexin V binding, and 7-AAD permeability in apoptotic cells. EL-4 cells were incubated with no treatment or with actinomycin D at 5 µg/mL for 4 h, followed by labeling with PhiPhiLux-G_1D_2, APC-conjugated annexin V, and 7-AAD at 5 µg/mL. The **top two** dot plots show forward vs side scatter profiles for both untreated and drug-treated cells; the **next two** dot plots show APC-annexin V vs 7-AAD permeability profiles for the same cells. The **third row** of histograms show caspase activation distributions for all cells, not gated for any other parameters; the **fourth row** for 7-AAD-negative annexin V-negative cells, with no gating for scatter characteristics. Percentage caspase-positive cells are shown. Cell controls with no PhiPhiLux-G_1D_2 loading are shown in the open histogram peaks.


no treatment
actinomycin D 16 h
side scatter
SSC-H
FSC-H
forward scatter
APC-annexin V
FL4-H
FL3-H
7-AAD
all cells
3.2%
55.2%
Events
FL1-H
all scatter
7-AAD-negative
annexin V-negative
0.7%
21.7%
PhiPhiLux-G1D2 caspase 3

apparent number of caspase-positive cells that are DNA dye-negative. This exclusion should be kept in mind when analyzing these early apoptotic subsets.

3.8. Simultaneous Immunophenotyping

The protocol in **Subheading 3.7.** is very compatible with simultaneous antibody immunophenotyping of the "viable" subpopulation. For example, PE-conjugated antibodies against a marker of interest could be combined with PhiPhiLux-G_1D_2, 7-AAD and APC-annexin V labeling as a very stringent "filter" for the removal of dead cells from the phenotyping analysis. While a natural extension of this method would appear to be the immunophenotyping of early apoptotic cells (such as caspase-positive/7-AAD negative/annexin V-negative), this should be approached with caution (*see* **Note 7**).

3.9. Sample Results

Sample results for fluorogenic caspase substrate labeling are shown below. In all of the illustrated results, apoptosis was induced in EL-4 murine thymoma cells by treatment with the transcriptional inhibitor actinomycin D for 4 h. This cell cycle blocker rapidly induced apoptosis via the caspase 3 pathway in many rapidly dividing cell lines. **Figures 1–4** both illustrate the expected results for the individual components of the multiparametric cell death assay described above, and demonstrate how the simultaneous analysis of multiple cell death characteristics in a single assay gives a multidimensional picture of the total apoptotic process.

1. Fluorescence distribution of PhiPhiLux-G_1D_2 labeling: **Figure 1** illustrates the typical signal-to-noise ration between "viable" and apoptotic EL-4 cells labeled with the PhiPhiLux-G_1D_2 substrate (here without subsequent annexin V and DNA binding dye labeling). Drug-treated EL-4 cells were analyzed for forward vs side scatter, where easily distinguishable populations of "viable" and "apoptotic cells were distinguishable based on cell size and optical density. The total population, the scatter "viable," and scatter apoptotic populations were then gated for PhiPhiLux-G_1D_2 fluorescence. The caspase substrate was readily detectable in the fluorescein channel by flow cytometry, in this case on a Becton-Dickinson FACSCalibur. The substrate is largely nonfluorescent in the uncleaved state; signal-to-noise ratios of 1–3 log orders of magnitude are normally seen between "viable" and apoptotic cells loaded with PhiPhiLux-G_1D_2, making the discrimination of apoptotic cells unambiguous.

   Interestingly, the "viable" and apoptotic distribution based on scatter measurements does not strictly correlate with caspase activation. The scatter "viable" cells in fact have a significant percentage of caspase-positive cells, indicating that cells activate caspases prior to gross changes in scatter morphology. Even more interesting, the scatter apoptotic population has some caspase-negative cells. While

some of these cells may be advanced apoptotic or necrotic cells with diminished or degraded caspase activity, there may also be viable cells in this population. Previous studies have demonstrated that cells may undergo transient volume fluctuations as a very early apoptotic marker, well prior to caspase activation. These results indicate the importance of not gate-excluding cells from subsequent apoptotic analysis based on their apparently apoptotic scatter characteristics; this population may contain some of the earliest detectable apoptotic cells.

2. PhiPhiLux-G_1D_2 and 7-AAD labeling. **Figure 2** shows the addition of 7-AAD labeling to the PhiPhiLux-G_1D_2 assay. The cytogram at the top of the figure shows 7-AAD labeling versus caspase activation for drug-treated EL-4 cells. Even with only two probes for apoptosis, four distinct subpopulations are apparent: a "viable" population at lower left, a caspase-positive population that has not progressed to 7-AAD permeability (lower right), and a caspase-positive population that is permeable to 7-AAD (upper right). Interestingly, a fourth population is also apparent that is permeable to 7-AAD but has little caspase activity. These cells are probably necrotic or advanced apoptotics, where caspases have leaked out of the cells, or have been proteolytically digested. Another potential source of this population is cells that have undergone apoptosis in the incubation period following PhiPhiLux labeling but prior to flow analysis. Cells in this region demonstrate the importance of analyzing cells promptly at the completion of the assay, as apoptosis is still occurring.

   Although we included all cells based on scatter in this analysis, 7-AAD labeling can now be used to exclude the more advanced apoptotics for specific measurement of the earlier dying cells. The left-most histogram shows caspase activation for all cells, while the histogram on the right is gated for 7-AAD-negative cells. Caspase activation is therefore clearly occurring prior to 7-AAD permeability.
3. PhiPhiLux-G_1D_2, 7-AAD and APC-annexin V labeling. **Figure 3** shows simultaneous analysis of all three cell death phenotypes in a single assay. The top two cytograms show the scatter characteristics of untreated and drug-treated EL-4 cells. The next two cytograms shown APC-annexin V binding vs 7-AAD permeability, ungated for scatter. These two characteristics appear to occur at approximately the same time for this cell type, with almost no single-positive cells for either phenotype. The histograms below were then gated for either the entire population, or APC-annexin V and 7-AAD-negative fluorescence. As above, these "early" apoptotics negative for PS "flipping" and loss of membrane permeability have a significant component of caspase-positive cells.
4. Detection of multiple caspases by flow cytometry. As was described in **Subheading 3.6.**, the PhiPhiLux system can incorporate a number of both consensus peptides for different caspase specificities, and fluorochromes for flow cytometric detection. It is therefore possible to load cells with more than one PhiPhiLux reagent, if they possess specificity for different caspases, and if they can be spectrally distinguished from one another by flow cytometry. In **Fig. 4**, apoptotic EL-4 cells have been loaded with PhiPhiLux-L_1D_2, which incorporates a caspase 8 consensus peptide instead of caspase 3/7 ***(19)***, and PhiPhiLux-

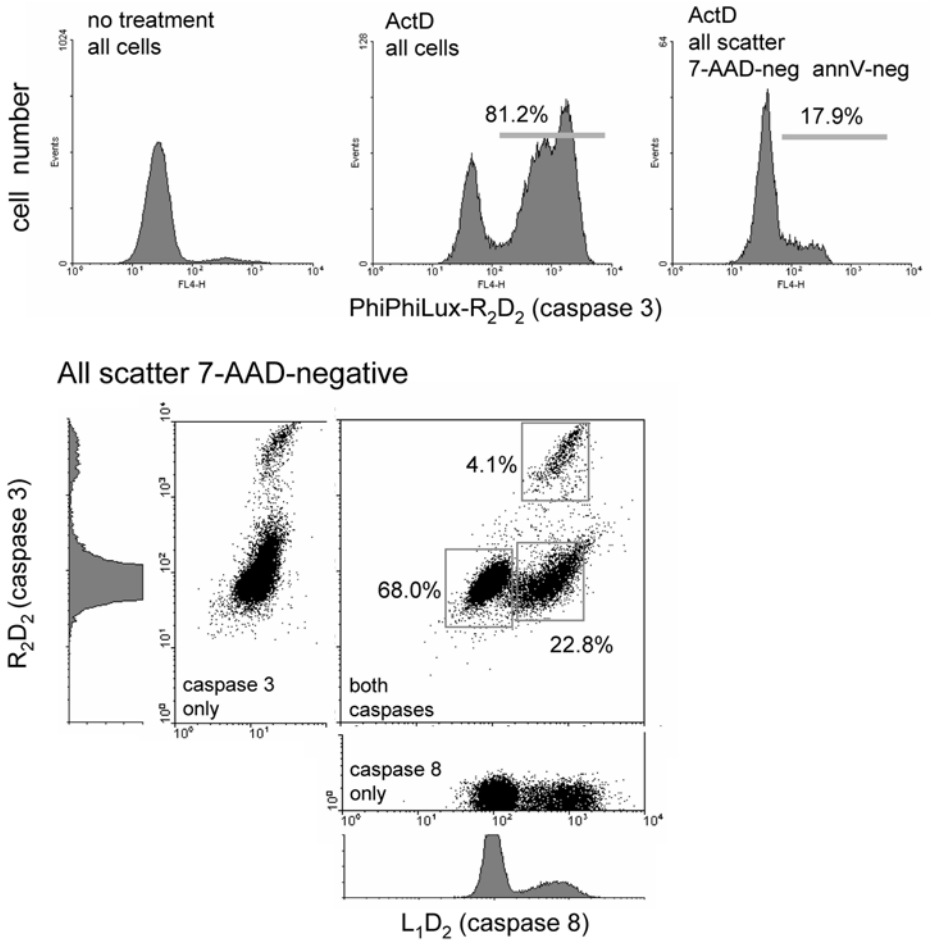


Fig. 4. Multiple caspase activation and 7-AAD permeability in apoptotic cells. EL-4 cells were incubated with no treatment or with actinomycin D at 5 µg/mL for 4 h, followed by labeling with PhiPhiLux-L_1D_2 (specific for caspase 8) and PhiPhiLux 3-R_2D_2 (specific for caspase 3), PE-conjugated annexin V and 7-AAD at 5 µg/mL. The **top row** of histograms shows caspase 3 activation distributions using the red-excited PhiPhiLux-3-R_2D_2 substrate for all untreated cells **(left)**, all drug-treated cells **(middle)**, and annexin V-negative 7-AAD-negative drug-treated cells **(right)**. The dot-plot **below** shows PhiPhiLux-L_1D_2 (caspase 8) vs PhiPhiLux-3-R_2D_2 (caspase 3) distributions for 7-AAD-negative cells (no scatter gate). Histograms of **plot fragments** on the **left** and **below** show single caspase substrate labeling controls. Percentages for caspase-positive populations are shown.

3-R_2D_2, which possesses the PARP consensus peptide and incorporates a proprietary Cy5-like red-excited fluorochrome. The cells were subsequently labeled with PE-conjugated annexin V and 7-AAD and analyzed on a dual-laser Becton-Dickinson FACSCalibur flow cytometer. The three histograms at the top of the figure show the caspase 3 activation profiles for untreated, all drug-treated, and annexin V-negative/7-AAD-negative drug-treated cells using this novel red-excited substrate; the profiles are similar to the PhiPhiLux-G_1D_2 reagent illustrated above. The dual caspase labeling experiment is shown in accompanying cytogram, gated for 7-AAD-negative cells. A caspase 8-positive caspase 3-negative population is clearly visible, consistent with previous biochemical observations that show caspase 8 activation to be necessary for caspase 3 activation. No caspase 3-positive caspase 8-negative population is present. This modification to the multiparametric cell death assay allows an even earlier stage of cell death to be distinguished and identified.

These collective results are consistent with many immune cell types and established cell lines; however, wider variations in apoptotic phenotype between different cell types should be expected (*see* **Note 8**).

4. Notes

1. Controls: Both "viable" and apoptotic controls are important for a meaningful analysis of apoptosis, and should be incorporated into any assay. If possible, an untreated negative control and an independent positive control should be included, the latter being induced by an agent other than that under study (such as a cytotoxic drug). Samples with both the absence and presence of the PhiPhiLux reagents are particularly important to include as controls, since the substrate does possess some low but detectable intrinsic fluorescence in the uncleaved state that can be erroneously interpreted as apoptosis without the appropriate control samples.
2. DNA binding dyes: Although PI and 7-AAD can be chosen based on their spectral characteristics, they are not completely interchangeable with regard to their permeability characteristics. 7-AAD is somewhat more cell permeable than PI; hence, it will give a greater percentage of apoptotic cells when compare directly to PI. This difference should be kept in mind while designing cell death assays, and may dictate the use of 7-AAD when this property is desired.
3. Multiparametric analysis of apoptosis in adherent cells: Flow cytometric analysis of apoptosis in adherent cell lines poses special challenges, as the removal of cells from their growth substrate may itself induce apoptosis. In addition, cell removal methods (such as trypsinization) can trigger false apoptotic indicators, such as aberrant annexin V binding in the absence of true cell death. By far the best solution to this problem is to utilize a laser scanning cytometer (LSC) for the analysis of apoptosis in these cell types; this specialized flow cytometer can perform "flow" analysis of cells on a flat surface, allowing minimal disruption during cell preparation ***(20)***. Several apoptosis assays, some utilizing caspase substrates have been described ***(16,21)***. The cell labeling protocol is similar to that for suspension

cells as described above, using cells cultured on tissue culture microslides as described previously ***(16)***. An example is shown in **Fig. 5**, where adherent L929 mouse fibroblasts were incubated with tumor necrosis factor-α (TNF-α) and cycloheximide, and labeled with PhiPhiLux-G_1D_2 and 7-AAD while still attached to their growth substrate. Analysis on a Compucyte laser scanning cytometer allowed easy discrimination of 7-AAD permeability and caspase activation, indicating that this assay is applicable to an adherent cell format as well.

4. Caspase substrate specificity and background. While the PhiPhiLux substrates seem reasonably specific for their target caspases, no synthetic substrate is exclusively specific for any particular enzyme. This should be kept in mind for any assay involving specific proteolytic activity. In general, a considerable excess of substrate will encourage low levels of nonspecific cleavage, increasing the non-caspase background of the assay. Titration of the substrate to the lowest concentration able to distinguish activity may be necessary when the specificity of the assay is in doubt.
5. Annexin V: The presence of calcium and magnesium is critical for annexin V binding; even removal of divalent cations after the binding reaction will result in rapid dissociation from PS residues. The cells must therefore remain in a calcium/magnesium buffer up to analysis.
6. Incubation periods: All incubation periods and conditions are critical parameters for this assay, as is prompt analysis of samples following the labeling procedure. Insufficient incubation time for the PhiPhiLux substrates will result in poor labeling; prolonged incubation periods will increase the level of nonspecific substrate binding and cleavage, resulting in high background fluorescence and decreased signal-to-noise ratios. In addition, prolonged storage of cells following removal of the surrounding PhiPhiLux substrate will eventually result in leakage of the cleaved substrate from the cell, despite its reduced cell permeability in the cleaved state. Overly long annexin V incubation periods will also increase the amount of nonspecific binding to cells, making discrimination of "viable" and apoptotic cells more difficult. Although PI (and to a lesser extent 7-AAD) are relatively impermeant to viable cells, prolonged incubation will cause uptake even in healthy cells. If laboratory conditions do not allow prompt analysis of sample, cell death assays involving fixed cells (such as TUNEL assays or immunolabeling of active caspases) should be considered as alternatives.
7. Simultaneous immunophenotyping of "viable" and early apoptotic cells: This method is readily amenable to the incorporation of antibody immunophenotyping along with the cell death markers, resulting in a very sophisticated "screening out" of dead cells for measurement of receptor expression in "viable cells." A potentially exciting extension of this method would appear to be the phenotyping of early apoptotic cells, positive for caspase expression but negative for later markers. This method should be approached with care; from a cellular standpoint, caspase activation is probably not an "early" event in cell death, and many alterations in the plasma membrane may have occurred by this timepoint, resulting in aberrant antibody binding to cells as is observed in later cell death. Any cell sur-

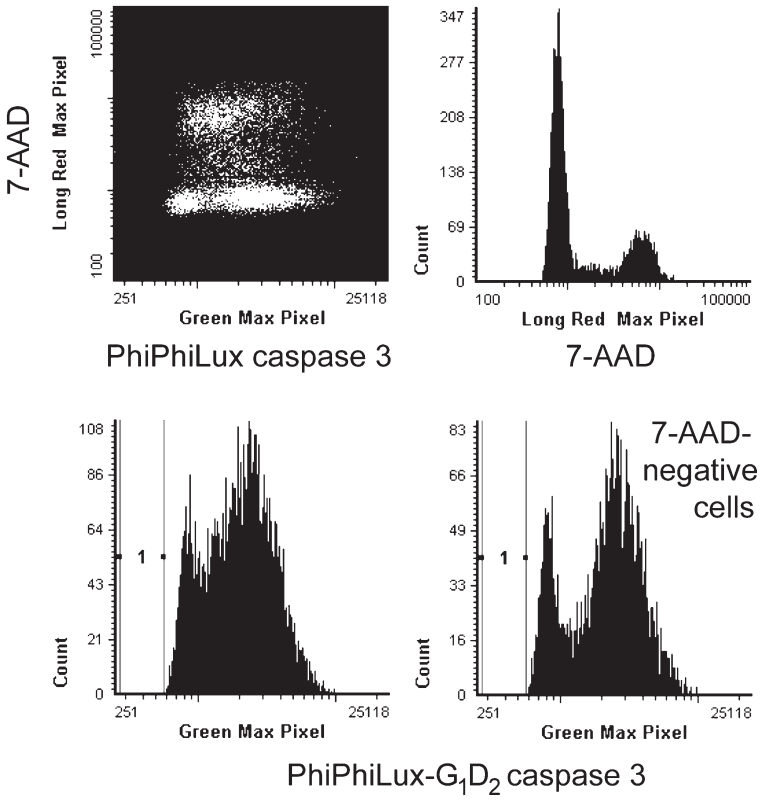


Fig. 5. Caspase activation and 7-AAD permeability in L929 fibroblasts by laser scanning cytometry. L929 cells were cultured on Nunc tissue culture microslides and incubated with TNF-α at 10 ng/mL and cycloheximide at 20 µg/mL for 8 h, followed by labeling with PhiPhiLux-G_1D_2 and 7-AAD at 5 µg/mL. Cells were then analyzed on a Compucyte laser scanning cytometer (Cambridge, MA) equipped with a 488-nm argon-ion laser. **Top left** dot-plot shows caspase 3 vs 7-AAD permeability, **top right** histogram shows 7-AAD permeability. The **lower** histograms show caspase activation distributions for all cells **(left)** and 7-AAD-negative cells **(right)**.

face marker expression results obtained by such methodology should be therefore be interpreted with caution.

8. Pleiotrophy in apoptosis: Apoptosis is a highly pleiotrophic process involving a variety of biochemical pathways; therefore, there are no universal morphological or physiological characteristics that are common to apoptosis in all cells. Cell death in different cell types (even in physiologically or morphologically similar ones) may present very different phenotypes, and may not necessarily be detectable by the same assays. Multiparametric assays for apoptosis are very amenable to this feature of apoptosis as the investigator is not limiting themselves

to one characteristic of cell death. However, the picture of cell death illustrated here may differ significantly in other tissues; this fact should be kept in mind.

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